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Isolation and characterization of streptomycin-resistant mutants in *Nicotiana plumbaginifolia*

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Summary. Streptomycin-resistant colonies were isolated from protoplast cultures of haploid *Nicotiana plumbaginifolia* based on their ability to green in medium containing 1 mg/ml streptomycin sulfate. The frequency of resistant colonies was 0.9×10^{-5} in nonmutagenized culture, and increased ten-fold following treatment of culture with 10 μg/ml N-methyl-N′-nitro-N-nitrosoguanidine. Of a total of 52 resistant clones isolated, 2 gave rise to haploid, 15 to diploid, and 3 to tetraploid plants upon transfer of calli to differentiation medium. Leaf-segment and protoplast assays showed that all diploid regenerates were resistant to streptomycin but sensitive to chloramphenicol, kanamycin, lincomycin, neomycin, and spectinomycin. Plants in most diploid clones were fertile and able to set seeds when self-fertilized and crossed reciprocally to wild-type plants. Inheritance of streptomycin resistance was studied in the diploid clones and, without exception, the resistance was transmitted maternally. Comparative studies of the ultrastructure of organelles and protein synthesis in isolated chloroplasts between wild-type and resistant clones in the presence of streptomycin suggest that streptomycin resistance is controlled by chloroplasts.

Key words: *Nicotiana plumbaginifolia* – Streptomycin resistance – Maternal inheritance – Chloroplast ultrastructure – In organello protein synthesis

Introduction

In higher plants, maternally inherited streptomycin-resistant mutants were first isolated from callus cultures of *Nicotiana tabacum* (Maliga et al. 1973, 1975; Umiel and Goldner 1976; Umiel 1979). Subsequent experiments in protoplast fusion have demonstrated that the resistance is controlled by chloroplasts (Menczel et al. 1981; Fluhr et al. 1983). Recently, more antibiotic-resistant mutants, including those resistant to streptomycin, lincomycin, spectinomycin, or chloramphenicol, were isolated from *N. tabacum* following treatment of seeds with N-nitroso-N-methylurea (Fluhr et al. 1985). Thus, abundant antibiotic-resistant mutants are now available in *N. tabacum*, providing useful markers for study of chloroplast genetics.

In contrast to the situation in *N. tabacum*, only lincomycin resistance (Cséplő and Maliga 1982, 1984) and triazine resistance (Cséplő et al. 1985) have been available as selectable chloroplast markers in *N. plumbaginifolia*. Since *N. plumbaginifolia* has been extensively used for investigation of chloroplast genetics and transfer (Cséplő et al. 1984; Medgyesy et al. 1985; Menczel et al. 1986), it is desirable that more plastome mutants be isolated from this species. In this paper, we report (1) a simple and efficient method for the isolation of streptomycin-resistant mutants from protoplast cultures of *N. plumbaginifolia*, and (2) morphological, cytological, biochemical, and genetic characteristics of these mutants.

Materials and methods

Plant material

Haploid plantlets of *Nicotiana plumbaginifolia* Viviani ($2n=20$) obtained from anther culture (Chen et al. 1985) were used. The plantlets were propagated by culturing lateral buds in a medium containing MS salts (Murashige and Skoog 1962), 1% sucrose, and 0.8% agar. The cultures were kept at 25°C under 16-h illumination with cool white fluorescent light of approximately 2000 lx.

Protoplast isolation and culture

The upper 2–3 fully expanded leaves of in vitro grown plantlets were used as the source of protoplasts. The methods for protoplast isolation and culture have been described elsewhere (Huang and Chen 1988).

Isolation of streptomycin-resistant plants

Freshly isolated protoplasts were suspended at a density of 10^5/ml in medium B (Huang and Chen 1988). After 2 days of culture in the dark at 25 °C, protoplast-derived cells were treated with 10 μg/ml N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) in the culture medium for 45 min, washed, and resuspended in the same medium. About 7–10 days after the mutagenic treatment, protoplast-derived colonies were plated at a density of 2×10^2/ml in medium C (Huang and Chen 1988) supplemented with 1 g/l casein hydrolysate, 0.5 g/l yeast extract, and 1 mg/ml streptomycin sulfate. The cultures were kept at 25 °C in the dark for 1 week and then placed under 16-h illumination of 2000 lx. Calli capable of greening were isolated and transferred onto medium D (Huang and Chen 1988) supplemented with 1 mg/ml streptomycin sulfate. Calli maintaining green on this medium were transferred for plant regeneration according to the method described by Huang and Chen (1988).

Leaf-segment assay for antibiotic resistance

The leaf blade was cut into small segments. The leaf segments were placed on a medium containing MS salts, 100 mg/l inositol, 1 mg/l thiamine-HCl, 0.5 mg/l α-naphthaleneacetic acid, 0.5 mg/l 6-benzylaminopurine, 3% sucrose, 0.8% agar, and one of the following antibiotics (Sigma): 500 μg/ml streptomycin sulfate, 50 μg/ml chloramphenicol, 50 μg/ml kanamycin monosulfate, 500 μg/ml lincomycin hydrochloride, 200 μg/ml neomycin sulfate, or 200 μg/ml spectinomycin dihydrochloride. The cultures were kept at 25 °C under 16-h illumination. Formation of green calli and/or shoots from the leaf segments was an indication of resistance.

Protoplast assay for streptomycin resistance

Protoplasts were isolated and cultured as described for isolation of streptomycin-resistant colonies. Formation of green colonies in the presence of streptomycin was indicative of resistance of the source plants.

Chromosome number determination

Roots were collected from plantlets grown in culture medium. They were treated with 0.002 *M* 8-hydroxyquinoline at 18°–20 °C for 3 h, fixed in ethanol-glacial acetic acid (3:1) overnight, stained by the Feulgen method, and then treated with 5% pectinase for 1 h. In the preparation of slides for chromosome counts, root tips were squashed in a drop of 45% acetic acid.

Estimation of pollen fertility

Anthers containing mature pollen grains were squashed in a drop of aceto-carmine. Only those grains which were plump and took up the stain were counted as viable. For each plant, more than 2000 pollen grains from two flowers were counted.

Seedling assay for streptomycin resistance

Seeds were sterilized in 1% sodium hypochlorite for 20 min and washed thoroughly with sterile distilled water. They were then germinated on a medium containing MS salts, 3% sucrose, 0.6% agar, and 1–5 mg/ml streptomycin sulfate. The cultures were incubated at 25 °C under 16-h illumination. Seedlings with green cotyledons were considered to be resistant while those with white cotyledons were sensitive.

Electron microscopy

Leaf calli which had been cultured for 4 weeks on medium D in the presence of 500 μg/ml streptomycin sulfate were fixed for 3 h at 4 °C in 2.5% glutaldehyde in 0.1 *M* phosphate buffer containing 5% sucrose (pH 7.0). After fixation, the samples were washed twice in the same buffer, treated with 1% osmium tetroxide for 2 h, and washed again in the buffer. The samples were dehydrated in a graded ethanol series, infiltrated, and embedded in Spurr's resin (Spurr 1969). Sections were stained with lead citrate and examined in a Hitachi H-600 transmission electron microscope.

Protein synthesis in isolated chloroplasts

Chloroplasts were isolated from young leaves according to the procedure of Fish and Jagendorf (1982). Aliquots of 26 μl of the chloroplasts (13–26 μg chlorophyll) were added to 78 μl of reaction buffer containing 35 m*M* Hepes-KOH (pH 8.3), 350 m*M* sorbitol, 300 μ*M* $MgCl_2$, 19 amino acids mixture (minus methionine) at 200 μ*M* each, and 1.1 m*M* dithiothreitol; 13 μl of [^{35}S]methionine (200 μCi, 1000 μCi/mmol; Amersham); and 13 μl of various concentrations of streptomycin sulfate. The chloroplast suspension was incubated for 1 h at 25 °C with illumination (3000 lx) and shaking (100 rpm). Aliquots of 10 μl of the suspension were removed for detection of radioactivity using a Beckman LS1801 Scintillation Counter.

Results

Streptomycin at 1 mg/ml slowed down cell division and suppressed greening of wild-type (sensitive) colonies. Colonies capable of greening and sustained growth in the presence of streptomycin were isolated and transferred again to medium containing the antibiotic. Those maintaining green after one subculture were considered to be resistant. The frequencies of streptomycin-resistant clones in control and mutagenized cultures are shown in Table 1. Treatment of protoplast-derived cells with MNNG resulted in a ten-fold increase in the frequency of resistant clones.

All 4 clones isolated from control culture and 16 out of 48 from mutagenized culture produced plants upon

Table 1. Frequencies of streptomycin-resistant clones in protoplast cultures of haploid *N. plumbaginifolia*

Mutagenic treatment	No. of colonies screened	No. of resistant clones	Frequency of resistant clones	Designation
None	4.6×10^5	4	0.9×10^{-5}	SR0001–SR0004
MNNG	5.0×10^5	48	9.6×10^{-5}	SR1001–SR1048

Table 2. Plant regeneration of streptomycin-resistant clones and ploidy levels of regenerated plants

Mutagenic treatment	No. of clones tested	No. of clones produced plants	Ploidy levels of regenerated plants			
			n	2*n*	3*n*	4*n*
None	4	4	2	2	0	0
MNNG	48	16	0	13	0	3

transfer to medium without auxin. Plants regenerated from each clone had the same number of chromosomes. The distribution of chromosome numbers of plants in the 20 clones is shown in Table 2. All clones were euploids, with diploids being predominant and haploids and polyploids relatively infrequent.

Tests of leaf segments on medium containing various antibiotics showed that all diploid regenerates were resistant to streptomycin (Fig. 1 a) but sensitive to chloramphenicol, kanamycin, lincomycin, neomycin, and spectinomycin. The streptomycin resistance was also confirmed by plating protoplast-derived colonies of a few representative plants in medium containing the antibiotic (Fig. 1 b).

The diploid regenerates were transplanted to pots and rasied to maturity. The color of the corolla of resistant plants was usually darker than that of wild type. Clones SR1012 and SR1021 had malformed flowers with short and twisted corolla. They also showed low pollen fertility and set seeds poorly when self-fertilized or crossed as male parent to wild-type plants. Clones SR1022 and SR1025 also contained low viable pollen, but like all other clones, they had normal flowers and were able to set seeds when self-fertilized and crossed reciprocally to wild-type plants. The percentages of pollen fertility of the 15 clones are listed in Table 3. Pollen fertility of wild-type plants was 86%.

Inheritance of streptomycin resistance of the diploid clones was investigated by germinating self-fertilized and F_1 seeds on medium containing 1 mg/ml streptomycin sulfate. The mode of inheritance of clones SR1012 and SR1021 was not clear because they were male sterile. However, seed set was possible when these clones were used as female parents, and all seedlings from the crosses showed streptomycin resistance. For all other clones, the resistant trait was transmitted only through the female parent (Fig. 1 c; Table 3).

The level of streptomycin resistance of the diploid clones was tested by germinating self-fertilized seeds or seeds from the cross of streptomycin-resistant ♀ × wild-type ♂ in the presence of 1–5 mg/ml streptomycin sulfate. Two classes of resistance were observed. The two clones isolated from control culture were resistant to streptomycin at 2 mg/ml, whereas those from mutagenized culture were resistant to 5 mg/ml.

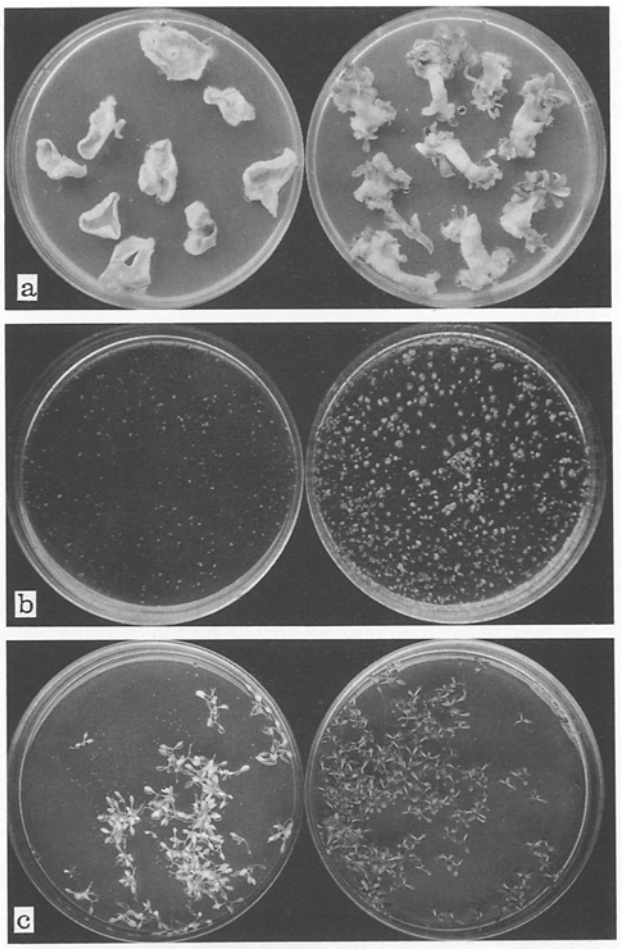

Fig. 1 a–c. Tests for streptomycin resistance. **a** Leaf-segment assay. Leaf segments from wild-type plants become bleached (left), whereas those from streptomycin-resistant clone SR1009 form green calli and shoots (right) in the presence of 500 µg/ml streptomycin sulfate. **b** Protoplast assay. Protoplast-derived colonies from wild type are bleached and unable to grow (left), whereas those from resistant clone SR1009 form green calli (right) in the presence of 1 mg/ml streptomycin sulfate. **c** Seedling assay. The cotyledons of seedlings from wild type♀ × SR1009♂ are white (left), whereas those from SR1009♀ × wild type♂ are green (right) in the presence of 1 mg/ml streptomycin sulfate

On medium with 500 µg/ml streptomycin sulfate, callus tissues derived from the leaves of wild-type plants became bleached, whereas those from resistant clones maintained green. Ultrastructurally, plastids in the bleached tissues showed abnormal external shape and contained no grana, but only rudimentary thylakoids and vesicules (Fig. 2 a). The changes in mitochondria were less conspicious. By contrast, chloroplasts and mitochondria in the green tissues of the resistant clones appeared quite normal. The grana in the chloroplasts

Table 3. Inheritance and pollen fertility of the diploid streptomycin-resistant clones of *N. plumbaginifolia*

Resistant clones	No. of resistant and sensitive seedlings[a]						Pollen fertility (%)
	SR♀ × wt♂		wt♀ × SR♂		SR selfed		
	R	S	R	S	R	S	
SR0002	79	0	0	67	217	0	83
SR0004	41	0	0	84	274	1	85
SR1007	135	0	0	219	97	0	59
SR1009	114	0	0	135	72	0	83
SR1012	117	0	–	–	–	–	9
SR1018	109	0	0	46	154	0	44
SR1019	115	0	0	139	146	0	64
SR1020	63	0	0	109	91	0	86
SR1021	98	0	–	–	–	–	15
SR1022	106	0	0	87	100	0	14
SR1025	127	0	0	109	112	0	22
SR1036	145	0	0	144	282	0	37
SR1037	88	0	1	341	144	0	51
SR1043	167	0	0	72	80	0	76
SR1046	162	0	0	235	78	0	82

[a] Seeds germinated on medium containing 1 mg/ml streptomycin sulfate

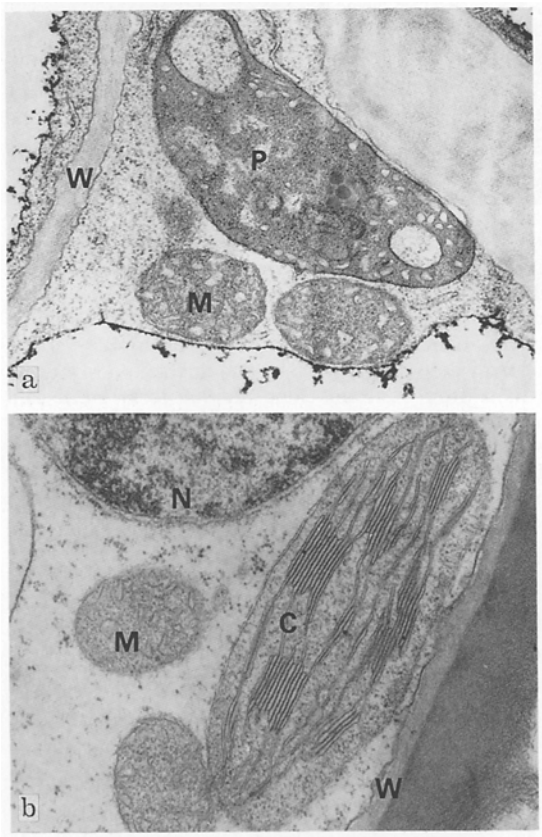

Fig. 2a and b. Electron micrographs showing chloroplasts and mitochondria in leaf callus of wild-type **a** and streptomycin-resistant clone SR1009 **b** cultured on medium with 500 μg/ml streptomycin sulfate. C – chloroplast; M – mitochondrion; N – nucleus; P – proplastid; W – cell wall. ×25,000

Table 4. Inhibition of streptomycin on protein synthesis in isolated chloroplasts

Streptomycin concentration (μg/ml)	Relative incorporation of [^{35}S]methionine (% of control)		
	wild type	SR1009	SR1019
0	100[a]	100[a]	100[a]
25	24	77	101
50	20	68	97
100	18	60	94
150	17	38	73
200	15	35	58

[a] In the absence of streptomycin, the incorporation for wild type, SR1009, and SR1019 is 1.35×10^7, 1.50×10^7, and 1.42×10^7 cpm/mg chlorophyll, respectively

were well-defined and consisted of up to ten thylakoids (Fig. 2b).

Chloroplasts were isolated from the wild-type and two streptomycin-resistant clones, SR1009 and SR1019, and the rate of incorporation of [^{35}S]methionine into newly synthesized proteins was compared. Virtually no incorporation was detected when the experiment was conducted in the dark (data not shown). Table 4 shows the relative incorporation of [^{35}S]methionine after 1-h incubation of chloroplasts under light. While in wild-type chloroplasts protein synthesis was reduced to 24% of the control by 25 μg/ml streptomycin sulfate, an equivalent inhibition was not obtained in chloroplasts from the resistant clones, even though the concentration of the antibiotic was increased eight-fold.

Discussion

The successful isolation of maternally inherited streptomycin-resistant mutants from protoplast cultures of *N. plumbaginifolia* may be attributed to two distinct features in the selection scheme. Firstly, application of selection pressure at an early stage of culture (9–12 days after culture initiation) provides a better opportunity for segregation and multiplication of the resistant organelles in dividing cells. In this regard, it is interesting to note that the resistant plants obtained in this study were homoplasmic, whereas those isolated by other methods often contained a mixed population of resistant and sensitive organelles (Cséplő and Maliga 1984; Fluhr et al. 1985). Secondly, addition of casein hydrolysate and yeast extract to the selective medium, which was originally designed to support the growth of auxotrophs (Sidorov et al. 1981), promotes greening of resistant colonies, thus enabling them to be recognized more easily among the bleached sensitive colonies.

Streptomycin resistance has also been shown to be the result of a recessive mutation in the nucleus (Sager 1954; Maliga 1981). In the present study, although haploid protoplasts were initially employed and about 90% protoplast-derived cells remained as haploids at the time of mutagenic treatment (Huang and Chen 1988; Chen et al. 1988), only cytoplasmic-resistant mutations were isolated. One possible explanation for failure of obtaining nuclear mutations is that the concentration of streptomycin (1 mg/ml) in the selective medium may not be suitable for selection of such mutations. In *Chlamydomonas,* it has been shown that nuclear mutations usually exhibit a lower level of resistance to streptomycin compared with chloroplast mutations (Sager 1954; Lee et al. 1973). Another possibility is that the samples used may not be large enough for screening nuclear mutations. Because of the polyploid nature of the chloroplast and mitochondrial genomes, it is expected that mutation would occur more frequently in the organelles than in the nucleus. In addition, it has been shown in *Chlamydomonas* that streptomycin is mutagenic for chloroplast DNA but not for nuclear DNA (Sager 1962).

The frequency of streptomycin-resistant colonies (0.9×10^{-5}) in nonmutagenized culture is considerably higher than the spontaneous streptomycin-resistant colonies (1×10^{-6}) in protoplast culture of *Onobrychis viciifolia* (Hamill et al. 1986), but lower than the lincomycin-resistant colonies (1×10^{-4}) in protoplast culture of *N. plumbaginifolia* (Cséplő and Maliga 1984). Treatment of culture with 10 μg/ml MNNG resulted in a ten-fold increase in the frequency of streptomycin-resistant colonies, which is comparable to the increase of lincomycin-resistant colonies induced by N-nitroso-N-ethylurea (Cséplő and Maliga 1984). Another effect of MNNG is a reduction in morphogenetic potential of callus (Table 2). However, the clones isolated from mutagenized culture showed a higher level of resistance to streptomycin compared with those from nonmutagenized culture. Mutations conferring resistance to different levels of streptomycin have also been found in *N. tabacum* (Fluhr et al. 1985) and *O. viciifolia* (Hamill et al. 1986).

Electron microscopic observations show that streptomycin affects chloroplast development in wild-type cells but does not appear to do so in the resistant mutants. A similar conclusions has been reached in *N. tabacum* (Maliga et al. 1975; Zamski and Umiel 1978) and *O. viciifolia* (Hamill et al. 1986). The effects of streptomycin on mitochondria have been rather controversial (Maliga et al. 1975; Zamski and Umiel 1978). In the present study, no conspicuous differences were observed between mitochondria of the wild type and those of the resistant mutants in the presence of 500 μg/ml streptomycin sulfate.

Further evidence linking streptomycin with the chloroplast was obtained from experiments of in organello protein synthesis. Our results show that protein synthesis in the assay system is light-dependent, indicating that it occurs in the chloroplasts and is not due to contamination of 80S cytoplasmic ribosomes. Thus, the resistance of mutant chloroplasts to streptomycin may be attributed to either an alteration of chloroplast ribosomes, which affects binding of the antibiotic, or to a change in chloroplast membrane proteins, which reduces uptake of the antibiotic.

The cross-resistance experiments show that the streptomycin-resistant mutants are sensitive to other aminoglycoside antibiotics such as kanamycin, neomycin, and spectinomycin. Similar results have also been obtained in one streptomycin-resistant line of *N. sylvestris* (Dix 1981). The reason(s) for this is not understood. However, it has been found in *E. coli* that each of the antibiotics mentioned above has a specific binding site in the 16S ribosomal RNA (Moazed and Noller 1987). We speculate that the streptomycin-resistant mutants obtained in this study may result from a change in the chloroplast 16S rRNA gene and that, as in *E. coli,* the binding site of streptomycin in 16S rRNA may be different from those of other aminoglycoside antibiotics. This speculation is consistent with the recent findings that streptomycin resistance in a number of eukaryotes including *N. tabacum* is caused by a single point mutation in the chloroplast 16S rRNA gene (Montandon et al. 1985; Harris et al. 1987; Etzold et al. 1987).

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References

Chen CC, Huang CR, To KY (1985) Anther cultures of four diploid *Nicotiana* species and chromosome numbers of regenerated plants. Bot Bull Acad Sin 26:147–153

Chen CC, Lee FM, Kao YY (1988) Endoreduplication in leaf protoplasts of haploid *Nicotiana plumbaginifolia* cultured in vitro. Genome 30:615–620

Cséplő Á, Maliga P (1982) Lincomycin resistance, a new type of maternally inherited mutation in *Nicotiana plumbaginifolia*. Curr Genet 6:105–109

Cséplő Á, Maliga P (1984) Large scale isolation of maternally inherited lincomycin resistance mutations, in diploid *Nicotiana plumbaginifolia* protoplast cultures. Mol Gen Genet 196:407–412

Cséplő Á, Nagy F, Maliga P (1984) Interspecific protoplast fusion to rescue a cytoplasmic lincomycin resistance mutation into fertile *Nicotiana plumbaginifolia* plants. Mol Gen Genet 198:7–11

Cséplő Á, Medgyesy P, Hideg E, Demeter S, Márton L, Maliga P (1985) Triazine-resistant *Nicotiana* mutants from photomixotrophic cell cultures. Mol Gen Genet 200:508–510

Dix PJ (1981) Cross-resistance in cell lines of *Nicotiana sylvestris* selected for resistance to individual antibiotics. Ann Bot 48:321–325

Etzold T, Fritz CC, Schell J, Schreier PH (1987) A point mutation in the chloroplast 16S rRNA gene of a streptomycin resistant *Nicotiana tabacum*. FEBS Lett 219:343–346

Fish LE, Jagendorf AT (1982) High rates of protein synthesis by isolated chloroplasts. Plant Physiol 70:1107–1114

Fluhr R, Aviv D, Edelman M, Galun E (1983) Cybrids containing mixed and sorted-out chloroplasts following interspecific somatic fusion in *Nicotiana*. Theor Appl Genet 65:289–294

Fluhr R, Aviv D, Galun E, Edelman M (1985) Efficient induction and selection of chloroplast-encoded antibiotic-resistant mutants in *Nicotiana*. Proc Natl Acad Sci USA 82:1485–1489

Hamill JD, Ahuja PS, Davey MR, Cocking EC (1986) Protoplast-derived streptomycin resistant plants of the forage legume *Onobrychis viciifolia* Scop. (sainfoin). Plant Cell Rep 5:439–441

Harris EH, Boyntol JE, Gillham NW (1987) Interaction of nuclear and chloroplast mutations in biogenesis of chloroplast ribosomes in *Chlamydomonas*. In: Wiessner E, Robinson DG, Starr RC (eds) Algal development. Molecular and cellular aspects. Springer Berlin Heidelberg New York, pp 142–149

Huang HC, Chen CC (1988) Genome multiplication in cultured protoplasts of two *Nicotiana* species. J Hered 79:28–32

Lee RW, Gillham NW, Van Winkle KP, Boynton JE (1973) Preferential recovery of uniparental streptomycin-resistant mutants from diploid *Chlamydomonas reinhardi*. Mol Gen Genet 121:109–116

Maliga P (1981) Streptomycin resistance is inherited as a recessive Mendelian trait in a *Nicotiana sylvestris* line. Theor Appl Genet 60:1–3

Maliga P, Sz-Breznovits Á, Márton L (1973) Streptomycin-resistant plants from callus culture of haploid tobacco. Nature New Biol 244:29–30

Maliga P, Sz-Breznovits Á, Márton L (1975) Non-Mendelian streptomycin-resistant mutants with altered chloroplasts and mitochondria. Nature 255:401–402

Medgyesy P, Fejes E, Maliga P (1985) Interspecific chloroplast recombination in a *Nicotiana* somatic hybrid. Proc Natl Acad Sci USA 82:211–215

Menczel L, Nagy F, Kiss Z, Maliga P (1981) Streptomycin-resistant and sensitive somatic hybrids of *Nicotiana tabacum* and *Nicotiana knightiana*: correlation of resistance to *Nicotiana* plastids. Theor Appl Genet 59:191–195

Menczel L, Polsby LS, Steinback KE, Maliga P (1986) Fusion-mediated transfer of triazine-resistant chloroplasts: Characterization of *Nicotiana tabacum* cybrid plants. Mol Gen Genet 205:201–205

Moazed D, Noller HF (1987) Interaction of antibiotics with functional sites in 16S ribosomal RNA. Nature 327:389–394

Montandon PE, Nicolas P, Schürmann P, Stutz E (1985) Streptomycin-resistance in *Euglena gracilis* chloroplasts: Identification of a point mutation in the 16S rRNA gene in an invariant position. Nucleic Acids Res 13:4299–4310

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Sager R (1954) Mendelian and non-Mendelian inheritance of streptomycin resistance in *Chlamydomonas reinhardi*. Proc Natl Acad Sci USA 40:356–363

Sager R (1962) Streptomycin as a mutagen for non-chromosomal genes. Proc Natl Acad Sci USA 48:2018–2026

Sidorov VA, Menczel L, Maliga P (1981) Isoleucine-requiring *Nicotiana* plant deficient in threonine deaminase. Nature 294:87–88

Spurr AR (1969) A low viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26:31–43

Umiel N (1979) Streptomycin resistance in tobacco. III. A test on germinating seedlings indicates cytoplasmic inheritance in St-R701 mutant. Z Pflanzenphysiol 92:295–301

Umiel N, Goldner R (1976) Effects of streptomycin on diploid tobacco callus cultures and the isolation of resistant mutants. Protoplasma 89:83–89

Zamski E, Umiel N (1978) Streptomycin resistance in tobacco. II. Effects of the drug on the ultrastructure of plastids and mitochondria in callus cultures. Z Pflanzenphysiol 88:317–325